MICROORGANISMS AND ASSAYS FOR THE IDENTIFICATION OF ANTIBIOTICS

Related Applications

The instant application claims the benefit of prior filed provisional U.S. Patent Application Serial No. 60/227,860, entitled "Novel Microbial Pantothenate Kinase Gene and Methods of Use", filed August 24, 2000. The instant application is also related to U.S. Patent Application Serial No. 09/667,569, entitled "Methods and Microorganisms for Production of Panto-Compounds", filed September 21, 2000 (pending). The entire content of the above-referenced patent applications is incorporated herein by this reference.

Background of the Invention

Antimicrobial or antibiotic treatment is a well-accepted therapy for fighting microbial infections that takes advantage of the existence of biological processes that are unique to bacteria or fungi, that can be safely inhibited to the detriment of the bacteria, without producing undesired or harmful side effects in the individual receiving such therapy. However, due at least in part to the continual evolution of microbial resistance to the available classes of antibiotics, and in part to the recent slowdown in the introduction of novel antimicrobials to market, there exists a need for the development of screening assays that target previously unexploited biochemical systems in microbes. In particular, there exists the need for the identification of new bacterial targets for use in drug discover programs designed to identify agents having potential use as anti-infective agents with novel modes of actions.

Summary of the Invention

The present invention is based at least in part, on the identification of a novel target for use in screening assays designed to identify antimicrobial agents. In particular, the present invention is based on the identification and characterization of a previously unidentified microbial pantothenate kinase gene, *coaX*. The *coaX* gene was first identified in *B. subtilis* where it is one of two genes encoding functional pantothenate kinase. Initially the present inventors identified and cloned the *B. subtilis coaA* gene (previously termed *yqjS*) that encodes a pantothenate kinase homologous to the CoaA enzyme previously characterized in *E. coli*. A second gene (previously termed *yacB*) has also been identified and cloned by the present

inventors that is not homologous to any previously described pantothenate kinase. This latter pantothenate kinase-encoding gene has been renamed *coaX*. The *coaX* gene could be deleted from *B. subtilis* strains with an intact *coaA* gene, but it could not be deleted from a strain containing a deletion in the *coaA* gene, indicating that the *coaX* gene is not essential in *B. subtilis* strains with a wild-type *coaA* gene. Homologs of the *coaX* gene can be found in a number of bacterial species, including but not limited to *Aquifex aeolicus, Bacillus anthracis, Bacillus halodurans, Bacillus stearothermophilus, Caulobacter crescentus, Chlorobium tepidum, Clostridium acetobutylicum, Dehalococcoides ethenogenes, Deinococcus radiodurans, Desulfovibrio vulgaris, Geobacter sulfurreducens, Pseudomonas putida, Rhodobacter capsulatus, Thiobacillus ferrooxidans, Streptomyces coelicolor, Synechocystis sp., Thermotoga maritima, Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Porphyromonas gingivalis, Pseudomonas aeruginosa, Pseudomonas syringae pv tomato, Treponema pallidum, Xylella fastidiosa* and *Mycobacterium tuberculosis*. More importantly, however, this novel pantothenate kinase gene has been found to be the sole essential pantothenate kinase in troublesome pathogens including, but not limited to, *Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Helicobacter pylori, Neisseria meningitidis, Pseudomonas aeruginosa, Treponema pallidum* and *Xylella fastidiosa*. Accordingly, the *coaX* gene represents an attractive target for screening for new antibacterial compounds to combat these pathogenic microorganisms, particularly microorganisms in which *coaX* is the sole pantothenate kinase-encoding gene.

Accordingly, the present invention features isolated CoaX proteins, in particular, proteins encoded by the *coaX* gene in bacteria. The invention also features isolated nucleic acid molecules and/or genes, *e.g.*, bacterial nucleic acid molecules and/or genes, in particular, isolated bacterial *coaX* nucleic acid molecules and/or genes. Also featured are vectors that contain isolated *coaX* nucleic acid molecules and/or genes as well as mutant *coaX* nucleic acid molecules and/or genes. Also featured are recombinant microorganisms (*e.g.*, microorganisms belonging to the genus *Escherchia* or *Bacillus*, for example, *E. coli* or *B. subtilis*) containing isolated *coaX* nucleic acid molecules and/or genes or mutant *coaX* nucleic acid molecules and/or genes of the present invention. In particular, the invention features recombinant microorganisms that produce the CoaX proteins of the present invention, *e.g.*, pantohthenate kinase proteins encodes by the *coaX* nucleic acid molecules and/or genes of the present invention.

Also featured are methods for identifying CoaX modulators utilizing, for example, isolated CoaX proteins of the present invention or recombinant microorganisms expressing the CoaX proteins of the present invention.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

Figure 1 is a schematic representation of the Coenzyme A biosynthetic pathway in *E. coli*.

Figure 2 is a schematic representation of the structure of the *Bacillus subtilis* genome in the region of the *coaA* gene. The scale is in base pairs and the significant open reading frames are shown by open arrows.

Figure 3 is a schematic representation of the structure of pAN296, a plasmid designed to delete most of the *B. subtilis coaA* gene and substitute a chloramphenicol resistance gene.

Figure 4 is a schematic representation of the structure of the *Bacillus subtilis* genome in the region of the *coaX* (*yacB*) gene. The scale is in base pairs, the significant open reading frames are shown by open arrows and certain predicted restriction fragments are indicated by thick bars.

Figure 5 is a schematic representation of the structure of pAN341 and pAN342, two independent PCR-derived clones of *B. subtilis yacB* (renamed herein as *coaX*).

Figure 6A-D depicts a multiple sequence alignment (MSA) of the amino acid sequences encoded by fourteen known or predicted microbial *coaX* genes. SEQ ID NOs:2-15 correspond to the amino acid sequences of *Bacillus subtilis* (SwissProt™ Accession No. P37564), *Clostridium acetobulyticum* (WIT™ Accession No. RCA03301, Argonne National Laboratories), *Streptomyces coelicolor* (PIR™ Accession No. T36391), *Mycobacterium tuberculosis* (SwissProt™ Accession No. O06282), *Rhodobacter capsulatus* (WIT™ Accession No. RRC02473), *Desulfovibrio vulgaris* (DBJ™ Accession No. BAA21476.1), *Deinococcus radiodurans* (SwissProt™ Accession No. Q9RX54), *Thermotoga maritima* (GenBank™ Accession No. AAD35964.1), *Treponema pallidum* (SwissProt™ Accession No. O83446), *Borrelia burgdorferi* (SwissProt™ Accession No.O51477), *Aquifex aeolicus* (SwissProt™ Accession No. O67753), *Synechocystis sp.* (SwissProt™ Accession No. P74045), *Helicobacter pylori* (SwissProt™ Accession No. O25533), and *Bordetella*

pertussis (SwissProt™ Accession No. Q45338), respectively. The alignment was generated using ClustalW MSA software at the GenomeNet CLUSTALW Server at the Institute for Chemical Research, Kyoto University. The following parameters were used: Pairwise Alignment, K-tuple (word) size = 1, Window size = 5, Gap Penalty = 3, Number of Top Diagonals = 5, Scoring Method = Percent; Multiple Alignment, Gap Open Penalty = 10, Gap Extension Penalty = 0.0, Weight Transition = No, Hydrophilic residues = Gly, Pro, Ser, Asn, Asp, Gln, Glu, Arg and Lys, Hydrophobic Gaps = Yes; and Scoring Matrix = BLOSUM.

Figure 7 is a schematic representation of the structure of pAN336, a plasmid designed to delete *B. subtilis coaX* from its chromosomal locus and replace it with a kanamycin resistence gene.

Figure 8 is a schematic representation of the construction of pOTP72, a plasmid containing the *H. pylori coaX* gene.

Figure 9 is a schematic representation of the construction of pOTP73, a plasmid containing the *P. aeruginosa coaX* gene.

Figure 10 is a schematic representation of the construction of pOTP71, a plasmid containing the *B. subtilis coaX* gene.

Detailed Description of the Invention

The present invention is based at least in part, on the identification of a novel target for use in screening assays designed to identify antimicrobial agents. In particular, the present invention is based on the identification and characterization of a previously unidentified microbial pantothenate kinase. This pantothenate kinase, encoded by a gene, termed *coaX* herein, is structurally unrelated to the previously characterized *E. coli* pantothenate gene, *coaA*, however, both genes encode functional pantothenate kinase enzymes, pantothenate kinase being essential for the synthesis of Coenzyme A (CoA). CoA is an essential coenzyme in all cells, participating in over 100 different intermediary reactions in cellular metabolism including, but not limited to, the tricarboxylic acid (TCA) cycle, fatty acid metabolism, vitamin biosynthesis and numerous other reactions of intermediary metabolism. Accordingly, pantothenate kinase production is essential for microbial growth. Coenzyme A (CoA) is synthesized in both eukaryotes and prokaryotes from pantothenate, also known as pantothenic acid or vitamin B5. The initial (and possibly rate-controlling) step in the conversion of pantothenate to Coenzyme A (CoA) is phosphorylation of pantothenate by pantothenate kinase. A schematic representation of the pathway leading to CoaA

biosynthesis in *E. coli*, *i.e.*, the *E. coli* CoA biosynthetic pathway is set forth as Figure 1. The term "CoA biosynthetic pathway", as used herein, includes the biosynthetic pathway involving CoA biosynthetic enzymes (*e.g.*, polypeptides encoded by biosynthetic enzyme-encoding genes), compounds (*e.g.*, precursors, substrates, intermediates or products), cofactors and the like utilized in the formation or synthesis of CoA from pantothenate. The CoA biosynthetic pathway depicted is also presumed to be that utilized by other microorganisms. The term "CoA biosynthetic pathway" includes the biosynthetic pathway leading to the synthesis of CoA in microorganisms (*e.g.*, *in vivo*) as well as the biosynthetic pathway leading to the synthesis of CoA *in vitro*.

The term "Coenzyme A or CoA biosynthetic enzyme" includes any enzyme utilized in the formation of a compound (*e.g.*, intermediate or product) of the CoA biosynthetic pathway, for example, the *coaA*, *panK* or *coaX* gene product which catalyzes the phosphorylation of pantothenate to form 4'-phosphopantothenate, or the *coaD* gene product which catalyzes the conversion of 4'-phosphopantetheine to dephosphocoenzyme A.

The *coaX* gene was first identified in *B. subtilis*, a microorganism in which it is one of two pantothenate kinase-encoding genes. Initially, the present inventors identified and cloned the *B. subtilis coaA* gene (previously termed *yqjS*) that encodes a pantothenate kinase homologous to the CoaA enzyme previously characterized in *E. coli*. A second gene (previously termed *yacB*) has also been identified and cloned by the present inventors that is not homologous to any previously described pantothenate kinase. This latter pantothenate kinase-encoding gene has been renamed *coaX*. The *coaX* gene could be deleted from *B. subtilis* strains with an intact *coaA* gene, but it could not be deleted from a strain containing a deletion in the *coaA* gene, indicating that the *coaX* gene is not essential in *B. subtilis* strains with a wild-type *coaA* gene.

Homologs of the *coaX* gene can be found in a number of bacterial species, including but not limited to *Aquifex aeolicus, Bacillus anthracis, Bacillus halodurans, Bacillus stearothermophilus, Caulobacter crescentus, Chlorobium tepidum, Clostridium acetobutylicum, Dehalococcoides ethenogenes, Deinococcus radiodurans, Desulfovibrio vulgaris, Geobacter sulfurreducens, Pseudomonas putida, Rhodobacter capsulatus, Thiobacillus ferrooxidans, Streptomyces coelicolor, Synechocystis sp., Thermotoga maritima, Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Porphyromonas gingivalis, Pseudomonas*

aeruginosa, Legionella pneumophila, Treponema pallidum, Xylella fastidiosa and *Mycobacterium tuberculosis*. More importantly, however, this novel pantothenate kinase gene has been found to be the sole essential pantothenate kinase in troublesome pathogens including, but not limited to, *Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Helicobacter pylori, Neisseria meningitidis, Pseudomonas aeruginosa, Treponema pallidum* and *Xylella fastidiosa*. Accordingly, the *coaX* gene represents an attractive target for screening for new antibacterial compounds to combat these pathogenic microorganisms, particularly microorganisms in which *coaX* is the sole pantothenate kinase-encoding gene.

Accordingly, in one aspect the present invention features assays for the identification an antibiotic that involve contacting a composition comprising a CoaX protein with a test compound; and determining the ability of the test compound to inhibit the activity of the CoaX protein; wherein the compound is identified as an antibiotic based on the ability of the compound to inhibit the activity of the CoaX protein. In another aspect, the invention features an assay for the identification a potential antibiotic that involves contacting an assay composition comprising CoaX with a test compound; and determining the ability of the test compound to bind to the CoaX; wherein the compound is identified as a potential antibiotic based on the ability of the compound to bind to the CoaX. In a preferred assay format, the composition is also contacted with pantothenate or a pantothenate analog and activity determined.

In another aspect, the invention features methods for identifying pantothenate kinase modulators that involve contacting a recombinant cell expressing a single pantothenate kinase encoded by a *coaX* gene with a test compound and determining the ability of the test compound to modulate pantothenate kinase activity in said cell. In another aspect, the invention features methods for identifying pantothenate kinase modulators that involve contacting a recombinant cell expressing a first and second pantothenate kinase, with a test compound and determining the ability of the test compound to modulate pantothenate kinase activity in said cell, wherein the first or second pantothenate kinase has reduced activity. Preferred recombinant microorganisms are of the genus *Bacillus* or *Escherchia* (*e.g.*, *Bacillus subtilis* or *Escherchia coli*).

Also featured are isolated nucleic acid molecules that include a *coaX* gene of the present invention, isolated proteins encoded by the *coaX* genes of the present invention and biologically active portions thereof. In one embodiment, the invention features a *coaX* gene derived from a microorganism selected from the group

consisting of *Aquifex aeolicus, Bacillus anthracis, Bacillus halodurans, Bacillus stearothermophilus, Bacillus subtilis, Caulobacter crescentus, Chlorobium tepidum, Clostridium acetobutylicum, Dehalococcoides ethenogenes, Deinococcus radiodurans, Desulfovibrio vulgaris, Geobacter sulfurreducens, Pseudomonas putida, Rhodobacter capsulatus, Thiobacillus ferrooxidans, Streptomyces coelicolor, Synechocystis sp., Thermotoga maritima, Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Porphyromonas gingivalis, Pseudomonas aeruginosa, Legionella pneumophila, Treponema pallidum, Xylella fastidiosa* and *Mycobacterium tuberculosis*, or a protein encoded by said *coaX* gene.

In another embodiment, the invention features isolated nucleic acid molecules that include a *coaX* gene derived from a pathogenic bacterium selected from the group consisting of *Bacillus anthracis, Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Pseudomonas aeruginosa, Porphyromonas gingivalis, Legionella pneumophila, Treponema pallidum* and *Xylella fastidiosa*, or a protein encoded by said *coaX* gene. In a preferred embodiment, the invention features isolated nucleic acid molecules that include a *coaX* gene derived from a pathogenic bacterium selected from the group consisting of *Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Helicobacter pylori, Neisseria meningitidis, Pseudomonas aeruginosa, Treponema pallidum* and *Xylella fastidiosa*, or a protein encoded by said *coaX* gene.

Also featured are recombinant vectors that include the isolated *coaX* genes of the present invention and recombinant microorganisms that include said vectors.

I. General Background

A pantothenate kinase activity was first identified in *Salmonella typhimurium* by screening for temperature-sensitive mutants which synthesized CoA at permissive temperatures but excreted pantothenate at non-permissive temperatures. The mutations were mapped in the *Salmonella* chromosome and the genetic locus was designated *coaA*. The gene encodes the enzyme that catalyzes the first step in the biosynthesis of coenzyme A from pantothenate (Dunn and Snell (1979) *J. Bacteriol.* 140:805-808). *Escherichia coli* temperature sensitive mutants have also been isolated and characterized (Vallari and Rock (1987) *J. Bacteriol.* 169:5795-5800). These mutants (named *coaA15(Ts)*) are defective in the conversion of pantothenate to CoA

and further exhibit a temperature-sensitive growth phenotype, indicating that pantothenate kinase activity is essential for growth. Moreover, it was noted that CoA inhibited pantothenate kinase activity to the same degree in the mutant as compared to the wild-type enzyme.

Feedback resistant *E. coli* mutants (named *coaA16(Fr)*) have also been isolated that possess a pantothenate kinase activity that is refractory to feedback inhibition by CoA (Vallari and Jackowski (1988) *J. Bacteriol.* 170:3961-3966). The mutation responsible for the reversion is, suprisingly, not genetically linked to the *coaA* gene by transduction. Additional data described therein support the view that the total cellular CoA content is controlled by both modulation of biosynthesis at the pantothenate kinase step and possibly by degradation of CoA to 4'-phosphopantetheine.

The wild-type *E. coli coaA* gene was cloned by functional complementation of *E. coli* temperature-sensitive mutants. The sequence of the wild-type gene was determined (Song and Jackowski (1992) *J. Bacteriol.* 174:6411-6417 and Flamm *et al.* (1988) *Gene* (*Amst.*) 74:555-558). Strains containing multiple copies of the *coaA* gene possessed 76-fold higher specific activity of pantothenate kinase, however, there was only a 2.7-fold increase in the steady state level of CoA (Song and Jackowski, *supra*). It has further been reported that the prokaryotic enzyme (encoded by *coaA* in *E.coli* and a variety of other microorganisms) is feedback inhibited by CoA both *in vivo* and *in vitro* with CoA being about five times more potent than acetyl-CoA in inhibiting the enzyme (Song and Jackowski, *supra* and Vallari *et al.*, *supra*). These data further support the view that feedback inhibition of pantothenate kinase activity is a critical factor controlling intracellular CoA concentration. The *E. coli* CoaA protein has been crystalized and the structure solved (Yun *et al.* (2000) *J. Biol. Chem.* 275(36):28093-28099).

Using standard search and alignment tools, *coaA* homologues have been identified in *Hemophilus influenzae*, *Mycobacterium tuberculosis*, *Vibrio cholerae*, *Streptococcus pyogenes* and *Bacillus subtilis*. By contrast, proteins with significant similarity could not be identified in eukaryotic cells including *Saccharomyces cerevisiae* or in mammalian expressed sequence tag (EST) databases. Using a genetic selection strategy, a cDNA encoding pantothenate kinase activity has recently been identified from *Aspergillus nidulans* (Calder *et al.* (1999) *J. Biol. Chem.* 274:2014-2020). The eukaryotic pantothenate kinase gene (*panK*) has distinct primary structure and unique regulatory properties that clearly distinguish it from its prokaryotic counterpart. A mammalian pantothenate kinase gene (*mpanK1α*) has also

been isolated which encodes a protein having homology to the *A. nidulans* PanK protein and to the predicted gene product of GenBank™ Accession Number 927798 identified in the *S. cerevisiae* genome (Rock *et al.* (2000) *J. Biol. Chem.* 275:1377-1383).

II. CoaX Nucleic Acid Molecules

The present invention relates, at least in part, to the identification of a novel microbial pantothenate kinase encoding gene, *coaX*, that is structurally distinct from a previously identified microbial pantothenate kinase encoding gene, *coaA*. Accordingly, one aspect of the present invention features isolated *coaX* nucleic acid molecules and/or genes useful, for example, for encoding pantothenate kinase enzymes for use in screening assays.

The term "nucleic acid molecule" includes DNA molecules (*e.g.*, linear, circular, cDNA or chromosomal DNA) and RNA molecules (*e.g.*, tRNA, rRNA, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA. The term "isolated" nucleic acid molecule includes a nucleic acid molecule that is free of sequences that naturally flank the nucleic acid molecule (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid molecule) in the chromosomal DNA of the organism from which the nucleic acid is derived. In various embodiments, an isolated nucleic acid molecule can contain less than about 10 kb, 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb, 0.1 kb, 50 bp, 25 bp or 10 bp of nucleotide sequences which naturally flank the nucleic acid molecule in chromosomal DNA of the microorganism from which the nucleic acid molecule is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular materials when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

The term "gene", as used herein, includes a nucleic acid molecule (*e.g.*, a DNA molecule or segment thereof), for example, a protein or RNA-encoding nucleic acid molecule, that in an organism, is separated from another gene or other genes, by intergenic DNA (*i.e.*, intervening or spacer DNA which naturally flanks the gene and/or separates genes in the chromosomal DNA of the organism). A gene may direct synthesis of an enzyme or other protein molecule (*e.g.*, may comprise coding sequences, for example, a contiguous open reading frame (ORF) which encodes a protein) or may itself be functional in the organism. A gene in an organism, may be clustered in an operon, as defined herein, said operon being separated from other

genes and/or operons by the intergenic DNA. Individual genes contained within an operon may overlap without intergenic DNA between said individual genes. An "isolated gene", as used herein, includes a gene which is essentially free of sequences which naturally flank the gene in the chromosomal DNA of the organism from which the gene is derived (*i.e.*, is free of adjacent coding sequences which encode a second or distinct protein or RNA molecule, adjacent structural sequences or the like) and optionally includes 5' and 3' regulatory sequences, for example promoter sequences and/or terminator sequences. In one embodiment, an isolated gene includes predominantly coding sequences for a protein (*e.g.*, sequences which encode *Bacillus* proteins). In another embodiment, an isolated gene includes coding sequences for a protein (*e.g.*, for a *Bacillus* protein) and adjacent 5' and/or 3' regulatory sequences from the chromosomal DNA of the organism from which the gene is derived (*e.g.*, adjacent 5' and/or 3' *Bacillus* regulatory sequences). Preferably, an isolated gene contains less than about 10 kb, 5 kb, 2 kb, 1 kb, 0.5 kb, 0.2 kb, 0.1 kb, 50 bp, 25 bp or 10 bp of nucleotide sequences which naturally flank the gene in the chromosomal DNA of the organism from which the gene is derived.

In one embodiment, an isolated nucleic acid molecule is or includes a *coaX* gene. In another embodiment, an isolated nucleic acid molecule is or includes a portion or fragment of a *coaX* gene. In one embodiment, an isolated *coaX* nucleic acid molecule is derived from a microorganism selected form the group consisting of *Aquifex aeolicus, Bacillus anthracis, Bacillus halodurans, Bacillus stearothermophilus, Bacillus subtilis, Caulobacter crescentus, Chlorobium tepidum, Clostridium acetobutylicum, Dehalococcoides ethenogenes, Deinococcus radiodurans, Desulfovibrio vulgaris, Geobacter sulfurreducens, Pseudomonas putida, Rhodobacter capsulatus, Thiobacillus ferrooxidans, Streptomyces coelicolor, Synechocystis sp., Thermotoga maritima, Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Porphyromonas gingivalis, Pseudomonas aeruginosa, Pseudomonas syringae pv tomato, Treponema pallidum, Xylella fastidiosa, Legionella pneumophila* and *Mycobacterium tuberculosis*. In another embodiment, an isolated *coaX* nucleic acid molecule is derived from a microorganism selected from the group consisting of *Bacillus anthracis, Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Porphyromonas gingivalis, Pseudomonas aeruginosa, Treponema pallidum, Xylella fastidiosa* and *Legionella pneumophila.* In another embodiment, an isolated *coaX* nucleic acid molecule is

derived from a microorganism selected from the group consisting of *Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Pseudomonas aeruginosa, Treponema pallidum* and *Xylella fastidiosa*. In another embodiment, an isolated *coaX* nucleic acid molecule or gene comprises a nucleotide sequence set forth as any one of SEQ ID NOs:SEQ ID NO:32, SEQ ID NO:69, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:1, SEQ ID NO:38, SEQ ID NO:31, SEQ ID NO:36, SEQ ID NO:52, SEQ ID NO:54, SEQ ID NO:23, SEQ ID NO:56, SEQ ID NO:58, SEQ ID NO:28, SEQ ID NO:60, SEQ ID NO:27, SEQ ID NO:34 or SEQ ID NO:68, SEQ ID NO:25, SEQ ID NO:40, SEQ ID NO:44, SEQ ID NO:42, SEQ ID NO:35 or SEQ ID NO:37, SEQ ID NO:62, SEQ ID NO:26, SEQ ID NO:24, SEQ ID NO:33, SEQ ID NO:29, SEQ ID NO:64, SEQ ID NO:30 and SEQ ID NO:66. In another embodiment, an isolated nucleic acid molecule of the present invention comprises a nucleotide sequence which is at least about 50-55%, preferably at least about 60-65%, more preferably at least about 70-75%, more preferably at least about 80-85%, and even more preferably at least about 90-95% or more identical to a nucleotide sequence set forth as any one of SEQ ID NOs:SEQ ID NO:32, SEQ ID NO:69, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:1, SEQ ID NO:38, SEQ ID NO:31, SEQ ID NO:36, SEQ ID NO:52, SEQ ID NO:54, SEQ ID NO:23, SEQ ID NO:56, SEQ ID NO:58, SEQ ID NO:28, SEQ ID NO:60, SEQ ID NO:27, SEQ ID NO:34 or SEQ ID NO:68, SEQ ID NO:25, SEQ ID NO:40, SEQ ID NO:44, SEQ ID NO:42, SEQ ID NO:35 or SEQ ID NO:37, SEQ ID NO:62, SEQ ID NO:26, SEQ ID NO:24, SEQ ID NO:33, SEQ ID NO:29, SEQ ID NO:64, SEQ ID NO:30 and SEQ ID NO:66.

In yet another embodiment, an isolated *coaX* nucleic acid molecule or gene comprises a nucleotide sequence that encodes a protein having an amino acid sequence as set forth in any one of SEQ ID NOs:SEQ ID NO:12, SEQ ID NO:70, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:2, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:3, SEQ ID NO:57, SEQ ID NO:8, SEQ ID NO:59, SEQ ID NO:7, SEQ ID NO:61, SEQ ID NO:6, SEQ ID NO:63, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:11, SEQ ID NO:21, SEQ ID NO:55, SEQ ID NO:14 or SEQ ID NO:67, SEQ ID NO:43 or SEQ ID NO:22, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:20, SEQ ID NO:10, SEQ ID NO:65 and SEQ ID NO:5. In yet another embodiment, an isolated *coaX* nucleic acid molecule or gene encodes a homologue of the CoaX proteins having the amino acid sequences of SEQ ID NOs:SEQ ID NO:12, SEQ ID NO:70, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:2, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:3, SEQ ID

NO:57, SEQ ID NO:8, SEQ ID NO:59, SEQ ID NO:7, SEQ ID NO:61, SEQ ID NO:6, SEQ ID NO:63, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:11, SEQ ID NO:21, SEQ ID NO:55, SEQ ID NO:14 or SEQ ID NO:67, SEQ ID NO:43 or SEQ ID NO:22, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:20, SEQ ID NO:10, SEQ ID NO:65 and SEQ ID NO:5. As used herein, the term "homologue" includes a protein or polypeptide sharing at least about 30-35%, preferably at least about 35-40%, more preferably at least about 40-50%, and even more preferably at least about 60%, 70%, 80%, 90% or more identity with the amino acid sequence of a wild-type protein or polypeptide described herein and having a substantially equivalent functional or biological activity as said wild-type protein or polypeptide. For example, a CoaX homologue shares at least about 30-35%, preferably at least about 35-40%, more preferably at least about 40-50%, and even more preferably at least about 60%, 70%, 80%, 90% or more identity with any one of the proteins having the amino acid sequences set forth as SEQ ID NOs:SEQ ID NO:12, SEQ ID NO:70, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:2, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:3, SEQ ID NO:57, SEQ ID NO:8, SEQ ID NO:59, SEQ ID NO:7, SEQ ID NO:61, SEQ ID NO:6, SEQ ID NO:63, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:11, SEQ ID NO:21, SEQ ID NO:55, SEQ ID NO:14 or SEQ ID NO:67, SEQ ID NO:43 or SEQ ID NO:22, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:20, SEQ ID NO:10, SEQ ID NO:65 and SEQ ID NO:5 and has a substantially equivalent functional or biological activity (*i.e.*, is a functional equivalent) of the proteins having the amino acid sequences set forth as SEQ ID NOs:SEQ ID NO:12, SEQ ID NO:70, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:2, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:3, SEQ ID NO:57, SEQ ID NO:8, SEQ ID NO:59, SEQ ID NO:7, SEQ ID NO:61, SEQ ID NO:6, SEQ ID NO:63, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:11, SEQ ID NO:21, SEQ ID NO:55, SEQ ID NO:14 or SEQ ID NO:67, SEQ ID NO:43 or SEQ ID NO:22, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:20, SEQ ID NO:10, SEQ ID NO:65 and SEQ ID NO:5 (*e.g.*, has a substantially equivalent CoaX activity). In a preferred embodiment, an isolated *coaX* nucleic acid molecule or gene comprises a nucleotide sequence that encodes a polypeptide as set forth in any one of SEQ ID NOs:SEQ ID NO:12, SEQ ID NO:70, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:2, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:3, SEQ ID NO:57, SEQ ID NO:8, SEQ ID NO:59, SEQ ID NO:7, SEQ ID NO:61, SEQ ID NO:6, SEQ ID NO:63, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:9, SEQ ID NO:15, SEQ ID

NO:11, SEQ ID NO:21, SEQ ID NO:55, SEQ ID NO:14 or SEQ ID NO:67, SEQ ID NO:43 or SEQ ID NO:22, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:20, SEQ ID NO:10, SEQ ID NO:65 and SEQ ID NO:5.

In another embodiment, an isolated *coaX* nucleic acid molecule hybridizes to all or a portion of a nucleic acid molecule having the nucleotide sequence set forth in any one of SEQ ID NOs:SEQ ID NO:32, SEQ ID NO:69, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:1, SEQ ID NO:38, SEQ ID NO:31, SEQ ID NO:36, SEQ ID NO:52, SEQ ID NO:54, SEQ ID NO:23, SEQ ID NO:56, SEQ ID NO:58, SEQ ID NO:28, SEQ ID NO:60, SEQ ID NO:27, SEQ ID NO:34 or SEQ ID NO:68, SEQ ID NO:25, SEQ ID NO:40, SEQ ID NO:44, SEQ ID NO:42, SEQ ID NO:35 or SEQ ID NO:37, SEQ ID NO:62, SEQ ID NO:26, SEQ ID NO:24, SEQ ID NO:33, SEQ ID NO:29, SEQ ID NO:64, SEQ ID NO:30 and SEQ ID NO:66 or hybridizes to all or a portion of a nucleic acid molecule having a nucleotide sequence that encodes a polypeptide having the amino acid sequence of any of SEQ ID NOs:SEQ ID NO:12, SEQ ID NO:70, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:2, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:3, SEQ ID NO:57, SEQ ID NO:8, SEQ ID NO:59, SEQ ID NO:7, SEQ ID NO:61, SEQ ID NO:6, SEQ ID NO:63, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:11, SEQ ID NO:21, SEQ ID NO:55, SEQ ID NO:14 or SEQ ID NO:67, SEQ ID NO:43 or SEQ ID NO:22, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:20, SEQ ID NO:10, SEQ ID NO:65 and SEQ ID NO:5. Such hybridization conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, Ausubel *et al.*, eds., John Wiley & Sons, Inc. (1995), sections 2, 4 and 6. Additional stringent conditions can be found in *Molecular Cloning: A Laboratory Manual*, Sambrook *et al.*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), chapters 7, 9 and 11. A preferred, non-limiting example of stringent hybridization conditions includes hybridization in 4X sodium chloride/sodium citrate (SSC), at about 65-70°C (or hybridization in 4X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 1X SSC, at about 65-70°C. A preferred, non-limiting example of highly stringent hybridization conditions includes hybridization in 1X SSC, at about 65-70°C (or hybridization in 1X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 0.3X SSC, at about 65-70°C. A preferred, non-limiting example of reduced stringency hybridization conditions includes hybridization in 4X SSC, at about 50-60°C (or alternatively hybridization in 6X SSC plus 50% formamide at about 40-45°C) followed by one or more washes in 2X SSC, at about 50-60°C. Ranges intermediate to the above-recited

values, *e.g.*, at 65-70°C or at 42-50°C are also intended to be encompassed by the present invention. SSPE (1X SSPE is 0.15 M NaCl, 10mM NaH_2PO_4, and 1.25 mM EDTA, pH 7.4) can be substituted for SSC (1X SSC is 0.15 M NaCl and 15 mM sodium citrate) in the hybridization and wash buffers; washes are performed for 15 minutes each after hybridization is complete. The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10°C less than the melting temperature (T_m) of the hybrid, where T_m is determined according to the following equations. For hybrids less than 18 base pairs in length, T_m(°C) = 2(# of A + T bases) + 4(# of G + C bases). For hybrids between 18 and 49 base pairs in length, T_m(°C) = 81.5 + 16.6($\log_{10}$[Na^+]) + 0.41(%G+C) - (600/N), where N is the number of bases in the hybrid, and [Na^+] is the concentration of sodium ions in the hybridization buffer ([Na^+] for 1X SSC = 0.165 M). It will also be recognized by the skilled practitioner that additional reagents may be added to hybridization and/or wash buffers to decrease non-specific hybridization of nucleic acid molecules to membranes, for example, nitrocellulose or nylon membranes, including but not limited to blocking agents (*e.g.*, BSA or salmon or herring sperm carrier DNA), detergents (*e.g.*, SDS), chelating agents (*e.g.*, EDTA), Ficoll, PVP and the like. When using nylon membranes, in particular, an additional preferred, non-limiting example of stringent hybridization conditions is hybridization in 0.25-0.5M NaH_2PO_4, 7% SDS at about 65°C, followed by one or more washes at 0.02M NaH_2PO_4, 1% SDS at 65°C, see *e.g.*, Church and Gilbert (1984) *Proc. Natl. Acad. Sci. USA* 81:1991-1995, (or, alternatively, 0.2X SSC, 1% SDS). In another preferred embodiment, an isolated nucleic acid molecule comprises a nucleotide sequence that is complementary to a *coaX* nucleotide sequence as set forth herein (*e.g.*, is the full complement of the nucleotide sequence set forth as SEQ ID NO:19). Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of SEQ ID NO:SEQ ID NO:32, SEQ ID NO:69, SEQ ID NO:46, SEQ ID NO:48, SEQ ID NO:50, SEQ ID NO:1, SEQ ID NO:38, SEQ ID NO:31, SEQ ID NO:36, SEQ ID NO:52, SEQ ID NO:54, SEQ ID NO:23, SEQ ID NO:56, SEQ ID NO:58, SEQ ID NO:28, SEQ ID NO:60, SEQ ID NO:27, SEQ ID NO:34 or SEQ ID NO:68, SEQ ID NO:25, SEQ ID NO:40, SEQ ID NO:44, SEQ ID NO:42, SEQ ID NO:35 or SEQ ID NO:37, SEQ ID NO:62, SEQ ID NO:26, SEQ ID NO:24, SEQ ID NO:33, SEQ ID NO:29, SEQ ID NO:64, SEQ ID NO:30 and SEQ ID NO:66, or to a complement thereof, corresponds to a naturally-occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature.

A nucleic acid molecule of the present invention (*e.g.*, a *coaX* nucleic acid molecule or gene), can be isolated using standard molecular biology techniques and the sequence information provided herein. For example, nucleic acid molecules can be isolated using standard hybridization and cloning techniques (*e.g.*, as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) or can be isolated by the polymerase chain reaction using synthetic oligonucleotide primers designed based upon the *coaX* nucleotide sequences set forth herein, or flanking sequences thereof. A nucleic acid of the invention (*e.g.*, a *coaX* nucleic acid molecule or gene), can be amplified using cDNA, mRNA or alternatively, chromosomal DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. Assays for identifying *coaX* gene of the present invention or homologues thereof can be accomplished, for example, by expressing the *coaX* gene in a microorganism, for example, a microorganism which expresses pantothenate kinase in a temperature-sensitive manner, and assaying the gene for the ability to complement a temperature sensitive (Ts) mutant for pantothenate kinase activity. A *coaX* gene that encodes a functional pantothenate kinase is one that complements the Ts mutant.

Yet another embodiment of the present invention features mutant *coaX* and *coaA* nucleic acid molecules or genes. The phrase "mutant nucleic acid molecule" or "mutant gene" as used herein, includes a nucleic acid molecule or gene having a nucleotide sequence which includes at least one alteration (*e.g.*, substitution, insertion, deletion) such that the polypeptide or protein that may be encoded by said mutant exhibits an activity that differs from the polypeptide or protein encoded by the wild-type nucleic acid molecule or gene. Preferably, a mutant nucleic acid molecule or mutant gene (*e.g.*, a mutant *coaA* or *coaX* gene) encodes a polypeptide or protein having a reduced activity (*e.g.*, having a reduced pantothenate kinase activity) as compared to the polypeptide or protein encoded by the wild-type nucleic acid molecule or gene, for example, when assayed under similar conditions (*e.g.*, assayed in microorganisms cultured at the same temperature). A mutant gene also can encode no polypeptide or have a reduced level of production of the wild-type polypeptide.

As used herein, a "reduced activity" or "reduced enzymatic activity" is one that is at least 5% less than that of the polypeptide or protein encoded by the wild-type nucleic acid molecule or gene, preferably at least 5-10% less, more preferably at least 10-25% less and even more preferably at least 25-50%, 50-75% or 75-100% less than that of the polypeptide or protein encoded by the wild-type nucleic acid molecule

or gene. Ranges intermediate to the above-recited values, *e.g.*, 75-85%, 85-90%, 90-95%, are also intended to be encompassed by the present invention. As used herein, a "reduced activity" or "reduced enzymatic activity" also includes an activity that has been deleted or "knocked out" (*e.g.*, approximately 100% less activity than that of the polypeptide or protein encoded by the wild-type nucleic acid molecule or gene). Activity can be determined according to any well accepted assay for measuring activity of a particular protein of interest. Activity can be measured or assayed directly, for example, measuring an activity of a protein isolated or purified from a cell. Alternatively, an activity can be measured or assayed within a cell or in an extracellular medium or in a crude extract of cells.

It will be appreciated by the skilled artisan that even a single substitution in a nucleic acid or gene sequence (*e.g.*, a base substitution that encodes an amino acid change in the corresponding amino acid sequence) can dramatically affect the activity of an encoded polypeptide or protein as compared to the corresponding wild-type polypeptide or protein. A mutant nucleic acid or mutant gene (*e.g.*, encoding a mutant polypeptide or protein), as defined herein, is readily distinguishable from a nucleic acid or gene encoding a protein homologue, as described above, in that a mutant nucleic acid or mutant gene encodes a protein or polypeptide having an altered activity, optionally observable as a different or distinct phenotype in a microorganism expressing said mutant gene or nucleic acid or producing said mutant protein or polypeptide (*i.e.*, a mutant microorganism) as compared to a corresponding microorganism expressing the wild-type gene or nucleic acid or producing said mutant protein or polypeptide. By contrast, a protein homologue has an identical or substantially similar activity, optionally phenotypically indiscernable when produced in a microorganism, as compared to a corresponding microorganism expressing the wild-type gene or nucleic acid. Accordingly it is not, for example, the degree of sequence identity between nucleic acid molecules, genes, protein or polypeptides that serves to distinguish between homologues and mutants, rather it is the activity of the encoded protein or polypeptide that distinguishes between homologues and mutants: homologues having, for example, low (*e.g.*, 30-50% sequence identity) sequence identity yet having substantially equivalent functional activities, and mutants, for example sharing 99% sequence identity yet having dramatically different or altered functional activities. Exemplary homologues are set forth as SEQ ID NOs:SEQ ID NO:12, SEQ ID NO:70, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:3, SEQ ID NO:57, SEQ ID NO:8, SEQ ID NO:59, SEQ ID NO:7, SEQ ID NO:61, SEQ ID

NO:6, SEQ ID NO:63, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:11, SEQ ID NO:21, SEQ ID NO:55, SEQ ID NO:14 or SEQ ID NO:67, SEQ ID NO:43 or SEQ ID NO:22, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:20, SEQ ID NO:10, SEQ ID NO:65 and SEQ ID NO:5 (*i.e.*, CoaX homologues). Exemplary mutants are described in Examples III-IV herein.

III. CoaX Proteins

Another aspect of the present invention features isolated proteins (*e.g.*, isolated CoaX proteins encoded, for example, by any one of the *coaX* genes or nucleic acids described herein). In one embodiment, the isolated proteins are produced by recombinant DNA techniques and can be isolated from microorganisms expressing, for example, any one of the *coaX* genes or nucleic acids described herein, by an appropriate purification scheme using standard protein purification techniques. In another embodiment, proteins are synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" protein (*e.g.*, an isolated or purified CoaX enzyme) is substantially free of cellular material or other contaminating proteins from the microorganism from which the protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. In one embodiment, an isolated or purified protein has less than about 30% (by dry weight) of contaminating protein or chemicals, more preferably less than about 20% of contaminating protein or chemicals, still more preferably less than about 10% of contaminating protein or chemicals, and most preferably less than about 5% contaminating protein or chemicals.

A "partially purified" protein (*e.g.*, a partially purified CoaX enzyme) is a compostion comprising a protein of interest where the composition has been subjected to at least one purification step, separation step, concentration step, or the like, such that the protein of interest is present at a greater concentration or level than prior to the purification step, separation step, concentration step, or the like. In one embodiment, a partially purified protein has between about 50-65% (by dry weight) of contaminating protein or chemicals, preferably between about 40%-50% of contaminating protein or chemicals, more preferably between about 30-40% of contaminating protein or chemicals.

Included within the scope of the present invention are CoaX proteins encoded by naturally-occurring bacterial or microbial genes, for example, by *coaX*

genes derived from a microorganism selected from the group consisting of *Aquifex aeolicus, Bacillus anthracis, Bacillus halodurans, Bacillus stearothermophilus, Bacillus subtilis, Caulobacter crescentus, Chlorobium tepidum, Clostridium acetobutylicum, Dehalococcoides ethenogenes, Deinococcus radiodurans, Desulfovibrio vulgaris, Geobacter sulfurreducens, Pseudomonas putida, Rhodobacter capsulatus, Thiobacillus ferrooxidans, Streptomyces coelicolor, Synechocystis sp., Thermotoga maritima, Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Porphyromonas gingivalis, Pseudomonas aeruginosa, Treponema pallidum, Xylella fastidiosa* and *Mycobacterium tuberculosis*. Further included within the scope of the present invention are CoaX proteins that are encoded bacterial or microbial genes which differ from naturally-occurring bacterial or microbial genes described herein, for example, genes which have nucleic acids that are mutated, inserted or deleted, but which encode proteins substantially similar to the naturally-occurring gene products of the present invention. For example, it is well understood that one of skill in the art can mutate (*e.g.*, substitute) nucleic acids which, due to the degeneracy of the genetic code, encode for an identical amino acid as that encoded by the naturally-occurring gene. Moreover, it is well understood that one of skill in the art can mutate (*e.g.*, substitute) nucleic acids which encode for conservative amino acid substitutions. It is further well understood that one of skill in the art can substitute, add or delete amino acids to a certain degree without substantially affecting the function of a gene product as compared with a naturally-occurring gene product, each instance of which is intended to be included within the scope of the present invention.

In one embodiment, an isolated protein of the present invention is encoded by a *coaX* gene derived from a microorganism selected from the group consisting of *Aquifex aeolicus, Bacillus anthracis, Bacillus halodurans, Bacillus stearothermophilus, Bacillus subtilis, Caulobacter crescentus, Chlorobium tepidum, Clostridium acetobutylicum, Dehalococcoides ethenogenes, Deinococcus radiodurans, Desulfovibrio vulgaris, Geobacter sulfurreducens, Pseudomonas putida, Rhodobacter capsulatus, Thiobacillus ferrooxidans, Streptomyces coelicolor, Synechocystis sp., Thermotoga maritima, Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Porphyromonas gingivalis, Pseudomonas aeruginosa, Treponema pallidum, Xylella fastidiosa* and *Mycobacterium tuberculosis*. In another embodiment, an isolated protein of the present invention is encoded by a

coaX gene derived from a microorganism selected from the group consisting of *Bacillus anthracis, Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Porphyromonas gingivalis, Pseudomonas aeruginosa, Legionella pneumophila, Treponema pallidum* and *Xylella fastidiosa* (*e.g.*, is encoded by a *coaX* gene derived from a pathogenic bacteria). In yet another embodiment, an isolated protein of the present invention is encoded by a *coaX* gene derived from a microorganism selected from the group consisting of *Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Pseudomonas aeruginosa, Treponema pallidum* and *Xylella fastidiosa* (*e.g.*, is encoded by a *coaX* gene derived from a pathogenic bacteria which has *coaX* as it's sole pantothenate kinase encoding enzyme). In a preferred embodiment, an isolated protein of the present invention (*e.g.*, a CoaX) has an amino acid sequence as set forth in any one of SEQ ID NOs:SEQ ID NO:12, SEQ ID NO:70, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:2, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:3, SEQ ID NO:57, SEQ ID NO:8, SEQ ID NO:59, SEQ ID NO:7, SEQ ID NO:61, SEQ ID NO:6, SEQ ID NO:63, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:11, SEQ ID NO:21, SEQ ID NO:55, SEQ ID NO:14 or SEQ ID NO:67, SEQ ID NO:43 or SEQ ID NO:22, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:20, SEQ ID NO:10, SEQ ID NO:65 and SEQ ID NO:5. In other embodiments, an isolated protein of the present invention (*e.g.*, a CoaX) is a homologue of the at least one of the proteins set forth as SEQ ID NOs:SEQ ID NO:12, SEQ ID NO:70, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:2, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:3, SEQ ID NO:57, SEQ ID NO:8, SEQ ID NO:59, SEQ ID NO:7, SEQ ID NO:61, SEQ ID NO:6, SEQ ID NO:63, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:11, SEQ ID NO:21, SEQ ID NO:55, SEQ ID NO:14 or SEQ ID NO:67, SEQ ID NO:43 or SEQ ID NO:22, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:20, SEQ ID NO:10, SEQ ID NO:65 and SEQ ID NO:5 (*e.g.*, comprises an amino acid sequence at least about 30-40% identical, preferably about 40-50% identical, more preferably about 50-60% identical, and even more preferably about 60-70%, 70-80%, 80-90%, 90-95% or more identical to the amino acid sequence of SEQ ID NOs:SEQ ID NO:12, SEQ ID NO:70, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:2, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:3, SEQ ID NO:57, SEQ ID NO:8, SEQ ID NO:59, SEQ ID NO:7, SEQ ID NO:61, SEQ ID NO:6, SEQ ID NO:63, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:9, SEQ ID

NO:15, SEQ ID NO:11, SEQ ID NO:21, SEQ ID NO:55, SEQ ID NO:14 or SEQ ID NO:67, SEQ ID NO:43 or SEQ ID NO:22, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:20, SEQ ID NO:10, SEQ ID NO:65 and SEQ ID NO:5, and has an activity that is substantially similar to that of the protein encoded by the amino acid sequence of SEQ ID NOs:SEQ ID NO:12, SEQ ID NO:70, SEQ ID NO:45, SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:2, SEQ ID NO:51, SEQ ID NO:53, SEQ ID NO:3, SEQ ID NO:57, SEQ ID NO:8, SEQ ID NO:59, SEQ ID NO:7, SEQ ID NO:61, SEQ ID NO:6, SEQ ID NO:63, SEQ ID NO:4, SEQ ID NO:13, SEQ ID NO:9, SEQ ID NO:15, SEQ ID NO:11, SEQ ID NO:21, SEQ ID NO:55, SEQ ID NO:14 or SEQ ID NO:67, SEQ ID NO:43 or SEQ ID NO:22, SEQ ID NO:39, SEQ ID NO:41, SEQ ID NO:20, SEQ ID NO:10, SEQ ID NO:65 and SEQ ID NO:5, respectively.

To determine the percent homology of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical positions/total # of positions x 100), preferably taking into account the number of gaps and size of said gaps necessary to produce an optimal alignment.

The comparison of sequences and determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-68, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-77. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Research* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g.,

XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller (1988) *Comput Appl Biosci.* 4:11-17. Such an algorithm is incorporated into the ALIGN program available, for example, at the GENESTREAM network server, IGH Montpellier, FRANCE (http://vega.igh.cnrs.fr) or at the ISREC server (http://www.ch.embnet.org). When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

In another preferred embodiment, the percent homology between two amino acid sequences can be determined using the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 12, 10, 8, 6, or 4 and a length weight of 2, 3, or 4. In yet another preferred embodiment, the percent homology between two nucleic acid sequences can be accomplished using the GAP program in the GCG software package (available at http://www.gcg.com), using a gap weight of 50 and a length weight of 3.

VI. Recombinant Nucleic Acid Molecules, Vectors and Microorganisms

The present invention further features recombinant nucleic acid molecules (*e.g.*, recombinant DNA molecules) that include nucleic acid molecules and/or genes described herein (*e.g.*, isolated nucleic acid molecules and/or genes), preferably pantothenate kinase-encoding genes (*e.g.*, *coaX* genes). The present invention further features vectors (*e.g.*, recombinant vectors) that include nucleic acid molecules (*e.g.*, isolated or recombinant nucleic acid molecules and/or genes) described herein. In particular, recombinant vectors are featured that include nucleic acid sequences that encode bacterial gene products as described herein, preferably bacterial nucleic acid sequences that encode bacterial pantothenate kinase proteins.

The term "recombinant nucleic acid molecule" includes a nucleic acid molecule (*e.g.*, a DNA molecule) that has been altered, modified or engineered such that it differs in nucleotide sequence from the native or natural nucleic acid molecule from which the recombinant nucleic acid molecule was derived (*e.g.*, by addition, deletion or substitution of one or more nucleotides). Preferably, a recombinant nucleic acid molecule (*e.g.*, a recombinant DNA molecule) includes an isolated nucleic acid molecule or gene of the present invention (*e.g.*, an isolated *coaX* gene) operably linked to regulatory sequences.

The term "recombinant vector" includes a vector (*e.g.*, plasmid, phage, phasmid, virus, cosmid or other purified nucleic acid vector) that has been altered, modified or engineered such that it contains greater, fewer or different nucleic acid sequences than those included in the native or natural nucleic acid molecule from which the recombinant vector was derived. Preferably, the recombinant vector includes a *coaX* gene or recombinant nucleic acid molecule including such *coaX* gene, operably linked to regulatory sequences, for example, promoter sequences, terminator sequences and/or artificial ribosome binding sites (RBSs), as defined herein.

The phrase "operably linked to regulatory sequence(s)" means that the nucleotide sequence of the nucleic acid molecule or gene of interest is linked to the regulatory sequence(s) in a manner which allows for expression (*e.g.*, enhanced, increased, constitutive, basal, attenuated, decreased or repressed expression) of the nucleotide sequence, preferably expression of a gene product encoded by the nucleotide sequence (*e.g.*, when the recombinant nucleic acid molecule is included in a recombinant vector, as defined herein, and is introduced into a microorganism).

The term "regulatory sequence" includes nucleic acid sequences which affect (*e.g.*, modulate or regulate) expression of other nucleic acid sequences. In one embodiment, a regulatory sequence is included in a recombinant nucleic acid molecule or recombinant vector in a similar or identical position and/or orientation relative to a particular gene of interest as is observed for the regulatory sequence and gene of interest as it appears in nature, *e.g.*, in a native position and/or orientation. For example, a gene of interest can be included in a recombinant nucleic acid molecule or recombinant vector operably linked to a regulatory sequence which accompanies or is adjacent to the gene of interest in the natural organism (*e.g.*, operably linked to "native" regulatory sequences, for example, to the "native" promoter). Alternatively, a gene of interest can be included in a recombinant nucleic acid molecule or recombinant vector operably linked to a regulatory sequence which accompanies or is adjacent to another (*e.g.*, a different) gene in the natural organism. Alternatively, a gene of interest can be included in a recombinant nucleic acid molecule or recombinant vector operably linked to a regulatory sequence from another organism. For example, regulatory sequences from other microbes (*e.g.*, other bacterial regulatory sequences, bacteriophage regulatory sequences and the like) can be operably linked to a particular gene of interest.

In one embodiment, a regulatory sequence is a non-native or non-naturally-occurring sequence (*e.g.*, a sequence which has been modified, mutated, substituted, derivatized, deleted including sequences which are chemically

synthesized). Preferred regulatory sequences include promoters, enhancers, termination signals, anti-termination signals and other expression control elements (*e.g.*, sequences to which repressors or inducers bind and/or binding sites for transcriptional and/or translational regulatory proteins, for example, in the transcribed mRNA). Such regulatory sequences are described, for example, in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in a microorganism (*e.g.*, constitutive promoters and strong constitutive promoters), those which direct inducible expression of a nucleotide sequence in a microorganism (*e.g.*, inducible promoters, for example, xylose inducible promoters) and those which attenuate or repress expression of a nucleotide sequence in a microorganism (*e.g.*, attenuation signals or repressor sequences). It is also within the scope of the present invention to regulate expression of a gene of interest by removing or deleting regulatory sequences. For example, sequences involved in the negative regulation of transcription can be removed such that expression of a gene of interest is enhanced.

In one embodiment, a recombinant nucleic acid molecule or recombinant vector of the present invention includes a nucleic acid sequence or gene that encodes at least one bacterial gene product (*e.g.*, a gene product encoded by *coaX*) operably linked to a promoter or promoter sequence. Preferred promoters of the present invention include *E. coli* promoters or *Bacillus* promoters and/or bacteriophage promoters (*e.g.*, bacteriophage which infect *E. coli* or *Bacillus*). In one embodiment, a promoter is a *Bacillus* promoter, preferably a strong *Bacillus* promoter (*e.g.*, a promoter associated with a biochemical housekeeping gene in *Bacillus* or a promoter associated with a glycolytic pathway gene in *Bacillus*). In another embodiment, a promoter is a bacteriophage promoter. In a preferred embodiment, the promoter is from the bacteriophage SP01. In a particularly preferred embodiment, a promoter is the P_{26} promoter set forth as SEQ ID NO:18 or the P_{15} promoter set forth as SEQ ID NO:19. Additional preferred promoters include *tef* (the translational elongation factor (TEF) promoter) and *pyc* (the pyruvate carboxylase (PYC) promoter), which promote high level expression in *Bacillus* (*e.g., Bacillus subtilis*). Additional preferred promoters, for example, for use in Gram positive microorganisms include, but are not limited to, the *amyE* promoter or phage SP02 promoters. Additional preferred promoters, for example, for use in Gram negative

microorganisms include, but are not limited to *tac, trp, tet, trp-tet, lpp, lac, lpp-lac, lacIq, T7, T5, T3, gal, trc, ara, SP6,* λ-P_R or λ-P_L.

In another embodiment, a recombinant nucleic acid molecule or recombinant vector of the present invention includes a terminator sequence or terminator sequences (*e.g.*, transcription terminator sequences). The term "terminator sequences" includes regulatory sequences which serve to terminate transcription of a gene. Terminator sequences (or tandem transcription terminators) can further serve to stabilize mRNA (*e.g.*, by adding structure to mRNA), for example, against nucleases.

In yet another embodiment, a recombinant nucleic acid molecule or recombinant vector of the present invention includes sequences which allow for detection of the vector containing said sequences (*i.e.*, detectable and/or selectable markers), for example, sequences that overcome auxotrophic mutations, for example, *trpC* or *leuB*, etc., fluorescent markers, and/or colorimetric markers (*e.g.*, *lacZ*/β-galactosidase), and/or antibiotic resistance genes (*e.g.*, *amp* or *tet*).

In yet another embodiment, a recombinant nucleic acid molecule or recombinant vector of the present invention includes an artificial ribosome binding site (RBS). The term "artificial ribosome binding site (RBS)" includes a site within an mRNA molecule (*e.g.*, coded within DNA) to which a ribosome binds (*e.g.*, to initiate translation) which differs from a native RBS (*e.g.*, a RBS found in a naturally-occurring gene) by at least one nucleotide. Preferred artificial RBSs include about 5-6, 7-8, 9-10, 11-12, 13-14, 15-16, 17-18, 19-20, 21-22, 23-24, 25-26, 27-28, 29-30 or more nucleotides of which about 1-2, 3-4, 5-6, 7-8, 9-10, 11-12, 13-15 or more differ from the native RBS (*e.g.*, the native RBS of a gene of interest). Preferably, nucleotides which differ are substituted such that they are identical to one or more nucleotides of an ideal RBS for a particular gene. Artificial RBSs can be used to replace the naturally-occurring or native RBS associated with a particular gene. Artificial RBSs preferably increase translation of a particular gene.

In another embodiment, a recombinant vector of the present invention includes sequences that enhance replication in bacteria (*e.g.*, replication-enhancing sequences). In one embodiment, replication-enhancing sequences are derived from *E. coli*. In another embodiment, replication-enhancing sequences are derived from pBR322.

In yet another embodiment, a recombinant vector of the present invention includes antibiotic resistance genes. The term "antibiotic resistance genes" includes sequences which promote or confer resistance to antibiotics on the host organism. In one embodiment, the antibiotic resistance genes are selected from the

group consisting of *cat* (chloramphenicol resistance) genes, *tet* (tetracycline resistance) genes, *amp* (ampicillin resistence), *erm* (erythromycin resistance) genes, *neo* (neomycin resistance) genes and *spec* (spectinomycin resistance) genes. Recombinant vectors of the present invention can further include homologous recombination sequences (*e.g.*, sequences designed to allow recombination of the gene of interest into the chromosome of the host organism). For example, *amyE* sequences can be used as homology targets for recombination into the host chromosome.

Preferred vectors of the present invention include, but are not limited to, vectors set forth in Figures 8-10. It will further be appreciated by one of skill in the art that the design of a vector can be tailored depending on such factors as the choice of microorganism to be genetically engineered, the level of expression of gene product desired and the like.

The methodologies of the present invention feature microorganisms, *e.g.*, recombinant microorganisms, preferably including genes or vectors as described herein, in particular, pantothenate kinase encoding genes or vectos. The term "recombinant" microorganism includes a microorganism (*e.g.*, bacteria, yeast cell, fungal cell, etc.) which has been genetically altered, modified or engineered (*e.g.*, genetically engineered) such that it exhibits an altered, modified or different genotype and/or phenotype (*e.g.*, when the genetic modification affects coding nucleic acid sequences of the microorganism) as compared to the naturally-occurring microorganism from which it was derived. Preferably, a "recombinant" microorganism of the present invention has been genetically engineered such that it overexpresses at least one bacterial gene or gene product (*e.g.*, a pantothenate kinase encoding gene) as described herein, preferably a pantothenate kinase encoding-gene included within a recombinant vector as described herein. The ordinary skilled will appreciate that a microorganism expressing or overexpressing a gene product produces or overproduces the gene product as a result of expression or overexpression of nucleic acid sequences and/or genes encoding the gene product.

The term "overexpressed" or "overexpression" includes expression of a gene product (*e.g.*, a pantothenate kinase) at a level greater than that expressed prior to manipulation of a microorganism or in a comparable microorganism that has not been manipulated. In one embodiment, a microorganism is genetically manipulated (*e.g.*, genetically engineered) to overexpress a level of gene product greater than that expressed prior to manipulation of the microorganism or in a comparable microorganism which has not been manipulated. Genetic manipulation can include,

but is not limited to, altering or modifying regulatory sequences or sites associated with expression of a particular gene (*e.g.*, by adding strong promoters, inducible promoters or multiple promoters or by removing regulatory sequences such that expression is constitutive), modifying the chromosomal location of a particular gene, altering nucleic acid sequences adjacent to a particular gene such as a ribosome binding site or transcription terminator, increasing the copy number of a particular gene, modifying proteins (*e.g.*, regulatory proteins, suppressors, enhancers, transcriptional activators and the like) involved in transcription of a particular gene and/or translation of a particular gene product, or any other conventional means of deregulating expression of a particular gene routine in the art (including but not limited to use of antisense nucleic acid molecules, for example, to block expression of repressor proteins).

In another embodiment, the microorganism can be physically or environmentally manipulated to overexpress a level of gene product greater than that expressed prior to manipulation of the microorganism or in a comparable microorganism which has not been manipulated. For example, a microorganism can be treated with or cultured in the presence of an agent known or suspected to increase transcription of a particular gene and/or translation of a particular gene product such that transcription and/or translation are enhanced or increased. Alternatively, a microorganism can be cultured at a temperature selected to increase transcription of a particular gene and/or translation of a particular gene product such that transcription and/or translation are enhanced or increased.

Still other preferred recombinant microorganisms of the present invention are mutant microorganisms. As used herein, the term "mutant microorganism" includes a recombinant microorganism that has been genetically engineered to express a mutated gene or protein that is normally or naturally expressed by the microorganism. Preferably, a mutant microorganism expresses a mutated gene or protein such that the microorganism exhibits an altered, modified or different phenotype (*e.g.*, has been engineered to express a mutated CoaA biosynthetic enzyme, for example, pantothenate kinase). In one embodiment, a mutant microorganism is designed or engineered such that it includes a mutant *coaX* gene, as defined herein. In another embodiment, a recombinant microorganism is designed or engineered such that it includes a mutant *coaA* gene, as defined herein. In another embodiment, a mutant microorganism is designed or engineered such that a *coaX* gene has been deleted (*i.e.*, the protein encoded by the *coaX* gene is not produced). In another embodiment, a mutant microorganism is designed or

engineered such that a *coaA* gene has been deleted (*i.e.*, the protein encoded by the *coaA* gene is not produced). Preferably, a mutant microorganism has a mutant *coaX* gene or a mutant *coaA* gene, or has been engineered to have a *coaX* gene and/or *coaA* deleted, such that that the mutant microorganism encodes a "reduced pantothenate kinase activity". In the context of a whole microorganism, pantothenate kinase activity can be determined by measuring or assaying for a decrease in an intermediate or product of the CoA biosynthetic pathway, for example, measuring or assaying for 4'-phosphopantothenate, 4'-phosphopantothenylcysteine, 4'-phosphopantetheine, dephosphocoenzyme A, Coenzyme A, apo-acyl carrier protein (apo-ACP) or holo-acyl carrier protein (ACP) in the microorganism (*e.g.*, in a lysate isolated or derived from the microorganism) or in the medium in which the microorganism is cultured. Alternatively, pantothenate kinase or CoaX activity can be determined by measuring or assaying for increased or decreased growth of the microorganism. Alternatively, pantothenate kinase activity can be determined indirectly by measuring or assaying for an increase in pantothenate which is the immediate precursor of pantothenate kinase.

In one embodiment, a recombinant microorganism of the present invention is a Gram negative organism (*e.g.*, a microorganism which excludes basic dye, for example, crystal violet, due to the presence of a Gram-negative wall surrounding the microorganism). In another embodiment, a recombinant microorganism of the present invention is a Gram positive organism (*e.g.*, a microorganism which retains basic dye, for example, crystal violet, due to the presence of a Gram-positive wall surrounding the microorganism). In a preferred embodiment, the recombinant microorganism is a microorganism belonging to a genus selected from the group consisting of *Escherichia, Heliobacter, Pseudomonas, Bordetella* and *Bacillus*. In a more preferred embodiment, the recombinant microorganism is of the genus *Escherichia* or *Bacillus*.

In another embodiment, the recombinant microorganism is a Gram negative (excludes basic dye) organism. In a preferred embodiment, the recombinant microorganism is a microorganism belonging to a genus selected from the group consisting of *Salmonella, Escherichia, Klebsiella, Serratia,* and *Proteus*. In a more preferred embodiment, the recombinant microorganism is of the genus *Escherichia*. In an even more preferred embodiment, the recombinant microorganism is *Escherichia coli*. In another embodiment, the recombinant microorganism is *Saccharomyces* (*e.g.*, *S. cerevisiae*).

V. Screening Assays

Because CoaX is an essential factor in bacteria, proteins (*e.g.*, enzymes) involved in the biosynthesis of CoA provide valuable tools in the search for novel antibiotics. In particular, the CoaX protein is a valuable target for identifying bacteriocidal compounds because it bears no resemblance in primary sequence to mammalian pantothenate kinase enzymes or CoaA's that are essential for beneficial enteric bacteria such as *E. coli*. Accordingly, the present invention also provides a method (also referred to herein as a "screening assay") for identifying modulators, *i.e.*, candidate or test compounds or agents (*e.g.*, peptides, peptidomimetics, small molecules or other drugs) that bind to CoaX, or have a stimulatory or inhibitory effect on, for example, *coaX* expression or CoaX activity.

In one embodiment, the invention provides assays for screening candidate or test compounds that are capable of binding to CoaX proteins or a biologically active portion thereof. In another embodiment, the invention provides assays for screening candidate or test compounds that modulate the activity of CoaX proteins or biologically active portions thereof. As used herein, the phrase "CoaX" activity includes any detectable or measurable activity of the CoaX protein, *i.e.*, the protein encoded by the *coaX* gene of the present invention, for example, the *coaX* gene derived from a microorganism selected from the group consisting of *Aquifex aeolicus, Bacillus anthracis, Bacillus halodurans, Bacillus stearothermophilus, Bacillus subtilis, Caulobacter crescentus, Chlorobium tepidum, Clostridium acetobutylicum, Dehalococcoides ethenogenes, Deinococcus radiodurans, Desulfovibrio vulgaris, Geobacter sulfurreducens, Pseudomonas putida, Rhodobacter capsulatus, Thiobacillus ferrooxidans, Streptomyces coelicolor, Synechocystis sp., Thermotoga maritima, Bordetella pertussis, Borrelia burgdorferi, Campylobacter jejuni, Clostridium difficile, Helicobacter pylori, Neisseria meningitidis, Neisseria gonorrhoeae, Porphyromonas gingivalis, Pseudomonas aeruginosa, Treponema pallidum, Xylella fastidiosa, Legionella pneumophila, and Mycobacterium tuberculosis*. In a preferred embodiment, a CoaX activity is at least one of the following: (1) modulation of at least one step in the CoA biosynthetic pathway; (2) promotion of CoA biosynthesis; (3) phosphorylation of a CoaX substrate; (4) a pantothenate kinase activity; and (4) complementation of a CoaX mutant.

The test compounds of the present invention can be obtained using any of the numerous approaches in chemical compound library methods known in the art, including: natural compound libraries; biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring

deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233. Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

In one embodiment, an assay is a microorganism-based assay in which a recombinant microorganism that expresses a CoaX protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to modulate CoaX activity is determined. Determining the ability of the test compound to modulate CoaX activity can be accomplished by monitoring, for example, growth, intracellular phosphopanthoate or CoA concentrations, or secreted pantothenate concentrations (as compounds that inhibit CoaX will result in a buildup of pantothenate in the test microorganism). CoaX substrate can be labeled with a radioisotope or enzymatic label such that modulation of CoaX activity can be determined by detecting a conversion of labeled substrate to intermediate or product. For example, CoaX substrates can be labeled with ^{32}P, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Determining the ability of a compound to modulate CoaX activity can alternatively be determined by detecting the induction of a reporter gene (comprising a CoA-responsive regulatory element operatively linked to a nucleic acid encoding a detectable marker, *e.g.*, luciferase), or detecting a CoA-regulated cellular response.

In yet another embodiment, a screening assay of the present invention is a cell-free assay in which a CoaX protein or biologically active portion thereof is contacted with a test compound *in vitro* and the ability of the test compound to bind to or modulate the activity of the CoaX protein or biologically active portion thereof is determined. In a preferred embodiment, the assay includes contacting the CoaX protein or biologically active portion thereof with known substrates to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to modulate enzymatic activity of the CoaX on its substrates.

Screening assays can be accomplished in any vessel suitable for containing the microorganisms, proteins, and/or reactants. Examples of such vessels include microtiter plates, test tubes, and micro-centrifuge tubes. In more than one embodiment of the above assay methods of the present invention, it may be desirable to immobilize either CoaX protein, CoaX substrate, substrate analogs or a recombinant microorganism expressing CoaX protein to facilitate separation of products, ligands, and/or substrates, as well as to accommodate automation of the assay. For example, glutathione-S-transferase/CoaX fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtiter plates. Other techniques for immobilizing proteins on matrices (*e.g.*, biotin-conjugation and streptavidin immobilization or antibody conjugation) can also be used in the screening assays of the invention.

This invention further pertains to novel agents identified by the above-described screening assays. Accordingly, it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example, a CoaX modulating agent identified as described herein (*e.g.*, an anti-bactericidal compound) can be used in an infectious animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent.

CoaX modulators can further be designed based on the crystal structure of any one of the CoaX proteins of the present invention. In particular, based at least in part on the discovery of CoaX as an essential bacterial protein, one can produce significant quantities of the CoaX protein, for example using the recombinant methodologies as described herein, purify and crystallize said protein, subject said protein to Xray crystallographic procedures and, based on the determined crystal structure, design modulators (*e.g.*, active site modulators, for example, competitor molecules, active site inhibitors, and the like), and test said designed modulators according to any one of the assays described herein.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

EXAMPLES

EXAMPLE I: Assays for CoaX genes or activities

Assay for pantothenate kinase genes or *in vivo* pantothenate kinase activity

In order to assay for genes encoding pantothenate kinase, the ability of plasmids containing these genes to complement the *coaA15(Ts15)* mutation in *E. coli* strain YH1 is tested at the non-permissive temperature of 43°-44°C. The defect in *E. coli coaA15(Ts)* has been identified as an S177L mutation that lies in a region that is highly conserved among bacterial pantothenate kinases, including CoaA of *B. subtilis*. Strain YH1 was constructed by P1 transduction from publically available strain DV62 (Coli Genetic Stock Center) to publically available strain YMC9 (ATCC), selecting for tetracycline resistance and screening for temperature sensitivity at 43°C.

In vitro assay for pantothenate kinase activity

The assay for pantothenate kinase is based on the fact that under appropriate mildly acidic conditions (1% acetic acid in 95% ethanol), the product of the reaction, 4'-phosphopantothenate, binds to positively charged ion exchange paper, while the substrate, pantothenate, does not (see Vallari, D., Jackowski, S., and Rock, C., (1987), Journal of Biological Chemistry, Vol. 262, pp2468-2471, hereby incorporated by reference).

Cells of the strain to be assayed (bacteria, yeast, fungi, animal, or plant cells) are grown to late logarithmic phase or stationary phase, in 200 ml of an appropriate medium, for example Luria Broth or M9 minimal salts plus 0. 5% glucose plus any necessary additives (for bacterial cells), at an appropriate temperature (25 to 44°C). All subsequent steps are carried out at 0 to 40°C. The culture is cooled on ice for 10 minutes and the cells are concentrated by centrifugation at 7,000 x g for 10 minutes. The cell pellet is rinsed by resuspending it in ice cold Buffer A (50 mM Tris-HCl, pH 7.4, 2.5 mM $MgCl_2$) and recentrifugation.

The rinsed cells are resuspended in the minimum possible volume (2-5 ml, depending on the size of the pellet) of Buffer A. The cells are then broken open by sonication in an inverted stainless steel test tube cap on ice for four bursts of 15 seconds each with 30 seconds of cooling between each burst. Cell debris is then

removed from the lysed cells by centrifugation at 10,000 x g for 10 minutes. The supernatant solution is then dialyzed for 12 - 16 hrs against two changes of one liter of Buffer A with 0.1 mM dithiothreitol added. Dialysis may be necessary to prevent the reaction product from undergoing further reactions catalyzed by the crude cell extract. Protein concentration in the dialyzed extracts is measured with a BCA Protein Assay Kit from BioRad.

The assay mix contains (final amounts or concentrations) about zero to 150 μg protein, 80 μM ^{14}C-D-pantothenate, specific activity about 60,000 dpm/nmole (purchased from American Radiolabeled Chemicals, Inc.), 2.5 mM ATP (Sigma Chemical Company, sodium salt), 2.5 mM MgCl2, and 100 mM Tris HCl, pH 7.4, in a total volume of 40 μl. The reaction mix, minus the ATP, can be preincubated for about 1 to 10 minutes at an appropriate temperature (25 to 55°C), in which case the reaction is started by addition of the ATP from a concentrated stock, also preincubated at the assay temperature.

After incubation for 1 to 10 minutes, the reaction is stopped by pipetting 35 μl of the reaction mix into an Eppendorf tube containing 1 ml of 95% ethanol, 1% acetic acid. After thorough mixing, the precipitated protein is pelleted in a microcentrifuge at top speed for one minute. The resulting supernatant solution is then applied to a one inch (or other appropriate size) disk of Whatman DE81 ion exchange filter paper prewetted with distilled water in a vacuum filtration manifold (for example Millipore 1225 Sampling Manifold). Each disk is then rinsed three times with 10 ml of 1% acetic acid in 95% ethanol. The top plate is then removed from the manifold and the completely exposed filter disks are each rinsed once more with 5 ml of the same rinse solution. The rinsed filters are then counted in a scintillation counter appropriatly set for ^{14}C in 10 ml of Ecolume scintillation fluid. The specific activity of the pantothenate kinase can be calculated by determining the number of moles of substrate converted to product per mg protein per minute under various appropriate conditions of the assay.

Appropriate assay blanks include, but are not limited to, the standard mix except without ATP or without protein extract, or a complete mix incubated on ice for the shortest possible time before pipetting to the filter disk (preferably less than 10 seconds).

The assay should be checked for linearity with time up to 10 minutes, and for linearity with protein between zero and 150 μg. No more than 10% of the

input 14C-pantothenate should be converted to phosphorylated product for the most accurate measurment of activity.

Temperature sensitivity of the pantothenate kinase enzyme can be tested by preincubating the reaction mix at various temperatures (25 to 55°C) for various lengths of time (zero to 60 minutes) before addition of ATP to start the reaction.

For pantothenate kinases other than that encoded by the *E. coli coaA* gene, the optimum temperature, pH, $MgCl_2$ concentration, buffering ion, ATP (or other substrate containing a high energy phosphate donor) concentration, salt type and concentration, total ionic strength, etc., may need to be determined. For accurate determination of enzyme activity, it may be necessary to purify or partially purify the pantothenate kinase enzyme from crude extracts, for example by ammonium sulfate fractionation and/or by column chromatography.

The assay may be adapted for high throughput screening, for example by using γ-thio-ATP instead of ATP and then reacting the transfered thio group with a conveniently detectable signalling molecule (see Jeong, S., and Nikiforov, T., (1999), Biotechniques Vol. 27, pp 1232-1238; and Facemyer, K., and Cremo, C., (1992), Bioconjug. Chem. Vol. 3, pp 408-413, both of which are hereby incorporated by reference).

EXAMPLE II: Identification and characterization of a first *B. subtilis* gene encoding pantothenate kinase, the *coaA* gene

The annotated version of the *B. subtilis* genome sequence available on the "Subtilist" web site contained no gene labeled as *coaA*. However a homology search using the protein sequence of *E. coli* pantothenate kinase as a query sequence gave a good match with *B. subtilis* gene *yqjS*, which is annotated as "unknown; similar to pantothenate kinase." This gene appears to be the penultimate gene in an operon containing five open reading frames (Figure 2). Two of the open reading frames encode proteins which are similar to D-serine dehydratase and to "ketoacyl reductase"; the other two have no known homologies. For the open reading frame corresponding to *coaA*, there are three possible start codons; each having a possible ribosome-binding site (RBS) associated with it. The three potential *coaA* ORFs were named *coaA1, coaA2,* and *coaA3*, from longest to shortest.

All three potential *coaA* open reading frames were cloned along with their respective RBSs by PCR followed by ligation into expression plasmid pAN229 to form plasmids pAN281, pAN282 and pAN283. pAN229 is a low copy vector in *E. coli* that provides expression from the SP01 phage P_{15} promoter and can integrate by single crossover at *bpr* with tetracycline selection.

To determine if the cloned putative *coaA* ORFs actually encode a pantothenate kinase activity, several isolates of all three plasmids were transformed into the *E. coli* strain YH1, that contains the *coaA15(Ts)* allele. Transformants were streaked to plates incubated at 30° and 43°C to test for complementation of the temperature sensitive allele. Isolates of all three *coaA* variants complemented well at 43°C, indicating that all three plasmid constructs encode an active pantothenate kinase. Accordingly, it can be concluded that the *B. subtilis yqjS* open reading frame codes for an active pantothenate kinase.

EXAMPLE III: Deletion of the *coaA* gene from the *B. subtilis* genome

The *coaA* gene of *B. subtilis* (*yqjS*) was deleted from the chromosome of a *B. subtilis* strain by conventional means. The majority of the *coaA* coding sequence was deleted from a plasmid clone and replaced by a chloramphenicol resistance gene (*cat*), while leaving approximately 1 kb of upstream and downstream sequence to allow homologous recombination with the chromosome, to give plasmid pAN296 (see Figure 3). pAN296 was then used to transform a *B. subtilis* strain (PY79), selecting for chloramphenicol resistance. The majority of transformants result from a double crossover event that effectively substitutes the *cat* gene for the *coaA* gene. The transformed strain containing the *coaA* deletion – *cat* insertion, named PA861) grew normally indicating the presence of a second *B. subtilis* pantothenate kinase encoding gene described herein.

EXAMPLE IV: Identification and characterization of a second *B. subtilis* gene encoding pantothenate kinase activity, the *coaX* gene

After finding that deletion of the *coaA* gene from the chromosome of *B. subtilis* is not a lethal event (see Example III), it was concluded that *B. subtilis* must contain a second gene that encodes an active pantothenate kinase, since pantothenate kinase is an essential enzyme activity.

A second pantothenate kinase-encoding gene was identified by complementing the *E. coli* strain YH1 (*coaA15(Ts)*) with a *B. subtilis* gene bank and selecting for transformants that were able to grow at 43°C. Found among the transformants were two families of plasmids that had overlapping restriction maps within each family, but not between the families. As expected, the restriction map of one family was identical to that predicted from the *B. subtilis* genome sequence for the homologue of the *E. coli coaA* gene (which we named *coaA* also, see above) and surrounding sequences. The other family had a restriction map that was completely non-overlapping with the first.

DNA sequencing of the ends of the cloned inserts from the second family showed that the clones came from a region of the *B. subtilis* chromosome that includes the 3' end of the *fts*H gene, the 5' end of the *sul* gene, and all of the *yacB, yacC, yacD, cysK, pabB, pabA* and *pabC* genes. None of the open reading frames of these cloned inserts showed homology to any known pantothenate kinase sequences, either prokaryotic or eukaryotic.

Several deletions were created through the *B. subtilis* genomic sequences in the cloned inserts. Each deletion was tested for complementation of the *E. coli* temperature sensitive pantothenate kinase. In particular, a deletion that removed all DNA between a *Stu* I site in the cloning vector and a *Swa* I site in the *yacC* gene, leaves *yacB* as the only intact open reading frame in the cloned insert (see Figure 4). This deleted plasmid still complemented the *E. coli* pantothenate kinase mutant. However, another deletion that removed DNA from the *Swa* I site in *yacC* through a *Bst*1107I site in the (already truncated) *ftsH* gene, could not complement the *E. coli* pantothenate kinase mutant. From these results, it was concluded that the *yacB* open reading frame was responsible for the complementation activity. To confirm that *yacB* is a pantothenate kinase gene, the *yacB* ORF plus 112 base pairs of downstream flanking sequence was amplified by PCR in two independent reactions and cloned downstream of a constitutive promote to give plasmids pAN341 and pAN342 (Figure 5). Both pAN341 and pAN342 complemented the defect in YH1 at 44°C, while a control plasmid, which has the same backbone, but expresses *panBCD* instead of *yacB* did not. This confirmed that the *yacB* open reading frame was responsible for the complementation of YH1.

As such, a novel gene that encodes pantothenate kinase activity in *B. subtilis* has been discovered that is not related by homology to any previously known pantothenate kinase gene. This gene has been renamed *coaX,* as a second, alternative

gene that encodes an enzyme that catalyzes the first step in the pathway from pantothenate to CoaA. In *B. subtilis* strains deleted for *coaA*, *coaX* is an essential gene.

Several homologues of the *B. subtilis coaX* gene were identified by homology searching of various publically available databases using the published *yacB* (*coaX*) open reading frame sequence and predicted amino acid sequence (as set forth in SEQ ID NOs:15 and 16 respectively). In two cases *(Mycobacterium tuberculosis and Streptomyces coelicolor)* the homologous *coaX* genes are adjacent to, or almost adjacent to, pantothenate biosynthetic genes, consistent with these homologs having a role in pantothenate metabolism. The CoaX proteins show no homology to the CoaA family of pantothenate kinases, nor to the eukaryotic family of pantothenate kinases exemplified by PanK of *Saccharomyces cerevisiae*.

Alignment of the amino acid sequences of several bacterial CoaX homologs with the amino acid sequence predicted from translating the *B. subtilis yacB* ORF described in the published *B. subtilis* genome sequence revealed that the CoaX proteins from other bacteria contained additional amino acid residues at their carboxy-terminal ends. Moreover, these extensions beyond the end of the predicted amino acid sequence for the *B. subtilis* gene product contained two relatively well conserved segments of sequence.

Translation of nucleotide sequences just downstream from the stop codon of the *B. subtilis yacB* ORF in a different reading frame revealed the existence of amino acid sequences very similar to the carboxy-terminal extensions of the other bacterial CoaX proteins. It is thus believed that an error exists in the published DNA sequence of the *B. subtilis yacB* ORF sequence that causes a frame shift leading to an artifactual downstream amino acid sequence and premature termination.

The PCR-generated sequences of *B. subtilis coaX* in pAN341 and pAN342 (described above) contain enough downstream flanking sequence to encode the putative carboxy-terminal extension described above, which is consistent with the result that the clones were functional in the complementation assay. However when the 3' PCR primer was positioned to include only the shorter *yacB* ORF predicted from the published sequence, but not to include the putative carboxy-terminal extension, then the resulting plasmids, pAN329 and pAN330 (similar in structure to pAN341 and pAN342; *see* Figure 5), did not complement the defect in YH1. This result supports the notion that the published *yacB* coding sequence contains a frame-shift error, and that the carboxy-terminal end of CoaX is necessary for pantothenate

kinase activity. A predicted correct nucleotide sequence for *B. subtilis coaX* is set forth as SEQ ID NO:1 and the translated amino acid sequence is set forth as SEQ ID NO:2. A multiple sequence alignment of the CoaX amino acid sequences of *B. subtilis* and 11 homologues thereof is set forth in Figure 6.

EXAMPLE V: Deleting the second pantothenate kinase gene, *coaX* gene from *B. subtilis*

With the knowledge gained above concerning the existence and nature of *coaX,* one can create a deletion of the *coaX* open reading frame from the *B. subtilis* chromosome that will remove the encoded activity, and that will not adversely affect the expression of the genes downstream from *coaX.* In such a deleted strain, the *coaA* gene will be the only gene that encodes pantothenate kinase.

To delete the *coaX* gene from *B. subtilis*, plasmid pAN336, which contains upstream and downstream homology for double crossover, was constructed with a kanamycin resistance gene replacing most of the *coaX* ORF (Figure 7). Strain PY79 was transformed to kanamycin resistance by pAN336, and an isolate confirmed to have resulted from a double crossover by PCR was named PA876. As predicted, deletion of *coaX* by itself is not lethal for *B. subtilis*. Furthermore, chromosomal DNA from PA876 would not transform competent PA861 (PY79 Δ*coaA ::cat*) to kanamycin resistance. These results indicate that it is the combination of Δ*coaA*::*cat* and Δ*coaX* :: *kan* that is lethal for *B. subtilis*, confirming that *B. subtilis* contains two unlinked genes that encode pantothenate kinase, *coaA* and *coaX*, and that either gene alone is capable of supplying sufficient pantothenate kinase for a normal rate of growth.

EXAMPLE VI: Identification of *coaX* homologs in other microbes

Database analyses reveal that many bacteria, in addition to *B. subtilis*, contain homologs of the CoaX pantothenate kinase. As shown in Tables 1 and 2, both nonpathogenic and pathogenic bacteria can be found that contain homologs of this novel gene.

Table 1: CoaX homologs in Non-Pathogens

Species	Genome complete	CoaA homolog	CoaX homolog
Aquifex aeolicus	Yes	NONE	RAA00700 aq_1924 AAC07720.1 pir||E70465
Bacillus halodurans	Yes	BH2875 BAB06594.1	BH0086
Bacillus stearothermophilus	No	NONE?	gnl|UOKNOR_1422|bstear_.Contig467
Bacillus subtilis	Yes	RBS02372 YqjS BAA12625.1 CAB14308.1 pir||C69965	RBS00070 YacB BAA05305.1 CAB11846.1 pir||S66100
Caulobacter crescentus	No	NONE?	gnl|TIGR|C.crescentus_12574
Chlorobium tepidum	No	NONE?	gnl|TIGR|C.tepidum_3499
Clostridium acetobutylicum	No	NONE?	RCA03301 gnl|GTC|C.aceto_gnl
Dehalococcoides ethenogenes	No	NONE?	gnl|TIGR_61435|deth_1587
Deinococcus radiodurans	Yes	NONE	AAF10040.1 pir||E75516
Desulfovibrio vulgaris	No	NONE?	BAA21476.1 P37564 gnl|TIGR_881|dvulg_1371
Geobacter sulfurreducens	No	NONE?	gnl|TIGR_35554|gsulf_121

***Pseudomonas putida* KT2440**	**No**	**NONE?**	**gnl|TIGR|pputida_10724**
Rhodobacter capsulatus	**No**	**NONE?**	**RRC02473**
Thiobacillus ferrooxidans	**No**	**NONE?**	**gnl|TIGR|t_ferrooxidans_6155**
Streptomyces coelicolor	**No**	**COAA_STRCO g8469186 pir||T35567**	**SCE94.31c CAB40880.1**
Synechocystis sp.	**Yes**	**NONE**	**ORF_ID:slr0812 BAA18120**
Thermotoga maritima	**Yes**	**NONE**	**TM0883 AAD35964.1 pir||D72320**

Table 2: CoaX homologs in Pathogens

Pathogen	Genome complete	CoaA homolog	CoaX homolog	Comments
Haemophilus influenzae	Yes	RHI13313	NONE	
Streptococcus pyogenes	No	RST01295	NONE	
Yersinia pestis	No	RYP02180	NONE	
Vibrio cholerae	Yes	VC0320	NONE	
Bacillus anthracis	No	NONE?	YES	
Bordetella pertussis	No	NONE?	BAF (BVG ACCESSORY FACTOR)	
Borrelia burgdorferi	Yes	NONE	BB0527	
Campylobacter jejuni	Yes	NONE	Cj0394c	
Clostridium difficile	No	NONE?	YES	
Helicobacter pylori	Yes	NONE	jhp0796 (strain J99) HP0862 (strain 26695) AAD07916.1	
Neisseria meningitidis	Yes	NONE	NMA0357 (strain Z2491) NMB2075 (strain MC58)	CoaX is fused to BirA
Neisseria gonorrhoeae	No	NONE?	RNG00193	CoaX is fused to BirA
Porphyromonas gingivalis	No	NONE?	RPG01037 gnl|TIGR|P.gingivalis_GPG.con	
Pseudomonas aeruginosa	Yes	NONE	RPA06755 PA4279 AAG07667.1	
Treponema pallidum	Yes	NONE	RTP00155 (TP0431)	
Xylella fastidiosa	Yes	NONE	XF1795	
Legionella	No		gnl|CUCGC_446|lpneumo	

pneumophila			**_C030598.2F12.S**	
Mycobacterium leprae	**No**	**MLCB1222.23**		
Mycobacterium tuberculosis	**Yes**	**RMT04257**	**RMT02984 (Rv3600c)**	**RMT04257**

Of particular interest are the seven human pathogens *Helicobacter pylori, Borrelia burgdorferi, Pseudomonas aeruginosa, Campylobacter jejuni, Neisseria meningitidis, Treponema pallidum*, and *Bordetella pertussis*, that contain the CoaX pantothenate kinase as their sole pantothenate kinase activity. For these bacteria, the CoaX pantothenate kinase represents an attractive target for screening for new antibiotics effective against one or more of these pathogens. One can overproduce the particular CoaX pantothenate kinase and use the isolated protein, partially purified protein or crude cell extracts to screen *in vitro* for compounds that modulate (*e.g.* inhibit) the pantothenate kinase activity. Alternatively, one can isolate compounds that specifically bind to the enzyme and test their ability to block the enzyme's activity. A known kinase activity represents a particularly favorable target for high-throughput screening for compounds that modulate or decrease that activity.

Also of interest are other pathogens which contain a *coaX* gene, in particular, if it is demonstrated that these other pathogens contain only a single pantothenate kinase encoded by the *coaX* gene. Examples of such bacteria are *Porphyromonas gingivalis, Neisseria gonorrhoeae, Clostridium difficile*, and *Bacillus anthracis*, all of which have been shown to contain a *coaX* homolog. Determination whether or not they also contain a second pantothenate kinase encoded by a *coaA* homolog can be determined according to the methodologies taught in Examples II-IV.

EXAMPLE VII: Identification of *coaX* homologs in human pathogens lacking a conventional prokaryotic pantothenate kinase

Human pathogens *Helicobacter pylori* (agent in gastoenteritus, stomach ulcers, and potentially stomach cancer), *Borrelia burgdorferi* (agent in Lyme's disease), *Bordetella pertussis* (agent in whooping cough), and *Pseudomonas aeruginosa* (opportunistic pathogen in cystic fibrous) all contain homologs of the *coaX* gene of *B. subtilis* and no homologs of the *coaA* gene of *E. coli* or *B. subtilis*. This is also true for the pathogens *Treponema pallidum*, *Campylobacter jejuni*, and *Neisseria meningitidis*. We have shown in *B. subtilis* that in the absence of the *coaA*

gene product (Δ*coaA* mutant), the *coaX* gene product is essential, providing the only pantothenate kinase activity required for the synthesis of the essential compound, Coenzyme A. Therefore it can be predicted that the pantothenate kinase encoded by the *coaX* homolog in the above listed pathogens is an essential enzyme for each mentioned pathogen and is required for the survival and growth of the pathogen. In fact it has been reported that the *coaX* homolog in *Bordetella pertussis*, called *baf*, and classified as an auxiliary regulatory factor rather than a critical enzyme, is an essential gene (see Wood, G.E. and R.L. Friedman (2000) FEMS Microbial. Lett. 193(1):25-30).

The CoaX protein is a favorable target for the development and screening of new antibiotics. First, the pantothenate kinase encoded by the *coaX* gene is an essential enzyme in a group of human pathogens, making it a good target for inactivation. Second, the enzyme activity (kinase) of the isolated CoaX protein or its homologs provides an ideal assay to screen large numbers of compounds (combinatorial libraries, etc.) for their ability to specifically inhibit the pantothenate kinase activity both *in vitro* and *in vivo*.

EXAMPLE VIII: Production of CoaX proteins from pathogens for use in screening assays.

To provide the pantothenate kinase proteins for screening assays, the *coaX* gene homolog was obtained by PCR from isolated, whole genome DNA of *Helicobacter pylori* (ATCC 700392), *Borrelia burgdorferi* (ATCC 35210), *Bordetella pertussis* (ATCC 9797), and *Pseudomonas aeruginosa* (ATCC 47085). Coding sequences for proteins with homology to *B. subtilis* CoaX were amplified by PCR using the primers and templates given in Table 3 with Pfx DNA polymerase (Life Technologies) according to the manufacture's specifications. The PCR primers incorporate a *XbaI* restriction enzyme recognition site at the 5' end of each product and a *BamHI* restriction enzyme recognition site at the 3' end of each product. PCR products were digested with a mixture of *XbaI* and *BamHI* and then purified by preparative agarose gel electrophoresis.

Table 3. PCR primers and template DNAs used to amplify coding sequences homologous to B. subtilis coaX.

Organism	***coaX* homolog**	**Template DNA**	**5' amplification primer**	**3' amplification primer**
Bacillus subtilis 168	*yacB*	Strain RL-1 genomic DNA	**TP175** 5'-GGGTCTAGAAAAGGAGGAA TTTAAATGTTACTGGTTATCGA TGTGGGGAACACC-3'	**TP176** 5'-GGGATCCTTATACACTTCCT ACGCGGTTTCTTTCATAAATC AATTCC-3'
Bordetella pertussis	*baf*	Strain ATCC 9797 genomic DNA	**TP177** 5'-GGGTCTAGAAAAGGAGGAA TTTAAATGATTATCCTCATCGA CTCCGGC-3'	**TP178** 5'-GGGATCCTTAGGCCGTTGG CGCGCCTTGCGCGGCG-3'
Borrelia burgdorferi	BB0527	Strain ATCC 35210 genomic DNA	**TP171** 5'-GGGTCTAGAAAAGGAGGAA TTTAAATGAATAAACCTTTATT ATCAGAATTGATAATTGATATT GGAAATACCAGC-3'	**TP172** 5'-GGGATCCTTAATTAACAAA CTTAAAGTCAATAGAATTTCC TAAAATTCTAACGCCTTCTAC AG-3'
Helicobacter pylori 26695	HP0862	Strain ATCC 700392 genomic DNA	**TP167** 5'-GGGTCTAGAAAAGGAGGAA TTTAAATGCCAGCTAGGCAATC TTTTACAGATTTGAAAAACCTG G-3'	**TP168** 5'-GGGATCCTTATTTGCATTCT AGTATCCCTGCTTTTTTAAGAG CGATTTCCATCCCGTC-3'
Pseudomonas aeruginosa PA01	PA4279	Strain ATCC 47085 genomic DNA	**TP169** 5'-GGGTCTAGAAAAGGAGGAA TTTAAATGATTCTTGAGCTCGA CTGTGGAAACTCGCTG-3'	**TP170** 5'-GGGATCCTTACTCAATCGG GCAAGCCAGTGCCAGCCCTAC G-3'

The purified PCR products were cloned by ligation with plasmid vector pASK-1BA3 (Sigma-Genosys) which had been digested with XbaI and BamHI followed by transformation into strains LH-1 and XL1-Blue/MRF'kan. Plasmids containing inserts were identified by restriction enzyme digestion of plasmid DNA isolated from selected transformants. Examples of plasmids containing the *H. pylori* (pOTP72), *P. aeruginosa* (pOTP73), or *B. subtilis* (pOTP71) *coaX* gene are shown in Figures 8, 9 and 10, respectively. The identity of inserts in plasmids is confirmed by DNA sequence analysis.

The pantothenate kinase activity of each of the above cloned *coaX* homologs can be demonstrated by transforming the plasmids described above into *E. coli* strain YH1 containing the *coaA15(Ts)* mutation and looking for complementation at the non-permissive temperature of 43°-44°C. For example, as shown in Table 4, transformation of *E. coli* YH1 containing the *coaA15(Ts)* with plasmid pOTP72 containing the cloned *H. pylori coaX* gene (HP0862) or plasmid pOTP73 containing the cloned *P. aeruginosa coaX* gene (PA4279) enabled the *E. coli* cells with the temperature sensitive *coaA* gene product to grow at 44°C as is also the case when these cells were transformed with the plasmid containing the *B. subtilis coaX* gene (pOTP71). These experiments confirm that the *coaX* homologs in *H. pylori* and *P. aeruginosa* due indeed each encode an active pantothenate kinase.

Table 4: Transformation of YH1 (coaA15(Ts)) with coaX ligation mixtures and control plasmid DNA

DNA	Number of colonies at 30°C	Number of colonies at 44°C
NONE	zero	zero
Ligated, cut vector	5	zero
Uncut vector (pASK-1BA3)	>500	zero
B. subtilis coaX, pool A ligation	74	67
B. subtilis coaX, pool B ligation	230	160
H. pylori coaX (HP0862) pool A	53	38

ligation		
H. pylori coaX (HP0862) pool B ligation	99	56
P. aeruginosa coaX (PA4279) pool A ligation	366	279
P. aeruginosa coaX (PA4279) pool B ligation	282	359

Since the *coaX* homologs cloned in pASK-1BA3 were inserted downstream of a Tet-inducible promoter, enzyme for *in vitro* screening assays can be obtained by inducing gene expression as described by Sigma-Genosys, and then isolating the overproduced pantothenate kinase by conventional protein purification procedure. Alternatively, the *coaX* gene can be cloned into any of various protein or peptide fusion expression vectors that facilitate purification of the protein. For example, *Helicobacter pylori, Borrelia burgdorferi, Bordetella pertussis*, and *Pseudomonas aeruginosa coaX* genes can be cloned into protein fusion expression vectors such as those available from companies including but not limited to Qiagen™ or Invitrogen™ to produce a His tagged CoaX fusion proteins or glutathione-S-transferase/CoaX fusion proteins which can be isolated by binding to nickel affinity or glutathione sepharose resins, respectively.

Equivalents Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.